eEF3: THE NEXT GREAT ANTIFUNGAL DRUG TARGET

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DEDICATION

To Dr. Dunaway,

thank you for the opportunity to contribute

to the research in your laboratory.

More importantly, thank you for believing in me

and helping me to believe in myself.

ABSTRACT

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The process of protein synthesis is crucial to the survival of every organism. Protein synthesis is accomplished by the ribosome, and consists of three major steps: initiation, elongation, and termination. The elongation step is carried out by two canonical elongation factors: eEF1A and eEF2. More recently, a third elongation factor was discovered: eEF3. eEF3 was determined to be fungal-specific; however, the results presented in this study suggest that conservation of eEF3 may be more widely distributed throughout the lower eukaryotic kingdom. The eEF3-like protein of *Phytophthora infestans* (PiEF3), an invasive oomycete, was cloned into *Saccharomyces cerevisiae* in order to determine if this protein could functionally complement for the loss of the endogenous eEF3. Based on results of cell viability, protein expression of PiEF3, and slight defects in protein synthesis, PiEF3 can support viability in *S. cerevisiae* as the sole eEF3 protein. Our data imply that eEF3 may be conserved beyond the fungal kingdom, thus challenging the four decade old notion that eEF3 is a fungal-specific protein. Moreover, understanding the distribution of eEF3 in lower eukaryotes contributes to a broader goal of developing an anti-eEF3 drug that should cause minimal collateral toxicity, given eEF3's absence in higher eukaryotic organisms.

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INTRODUCTION

Pathogens of the Fungal Kingdom

Animals and fungi shared a common ancestor one billion years ago, illustrating these lineages as sister kingdoms (Heitman 2011). The fungal kingdom continued to evolve into eight to ten phyla. While the fungal kingdom has proven its usefulness in the fields of research, medicine, and industry in various capacities, it has also been deemed an increasingly dangerous threat to mammalian health and agriculture (Brown et al. 2012).

Within the fungal lineages, pathogenic fungi have diverged repeatedly and independently, leading to many different virulence mechanisms among these pathogens. Specifically, many human and plant pathogens have arisen from the Ascomycota and Basidiomycota phyla. The most notorious pathogenic lineages within the Ascomycota are the *Pneumocystis, Candida* and *Aspergillus* species (Heitman 2011). *Pneumocystis jirovecii* is notorious for infecting the lungs of HIV/AIDS patients causing pneumonia. Additionally, although *Candida albicans* is a normal component of microflora and microbiota, it can opportunistically cause both mucocutaneous and cutaneous infections (Heitman 2011). In regard to the Basidiomycota phylum, the most threatening pathogen is *Cryptococcus neoformans. C. neoformans* is a fungal pathogen that causes lethal meningoencephalitis and is also particularly threatening to immunocompromised patients (Blakely et al. 2001). In addition to those with HIV/AIDS, immunocompromised patients also include those receiving chemotherapy, steroids, and organ transplantations. Overall, the severity of the fungal threat is most dangerous to immunocompromised patients, due to the ability of fungal pathogens to take advantage of the vulnerable immune system. Moreover, the severity is also due to the rapid emergence and evolution of these infectious diseases. The large

population size and rapid life cycles of these pathogens accounts for the rise of drug resistance, which parallels incidence rate (Brown et al. 2012).

The Emerging Mycoses Threat

Fungal Threats to Human Health

Despite a myriad of defense mechanisms provided by modern medical technology, invasive fungal infections, also known as mycoses, are rapidly emerging and pose a major health concern. The United States has suffered a drastic 320% increase in the annual number of deaths due to invasive mycosis between 1980 and 1997 (Low and Rotstein 2011). More recently, it has been reported that Aspergillosis infections, specifically allergic bronchopulmonary aspergillosis (ABPA), affect 4.8 million people worldwide (2.5% of adults) (CDC 2014). Of the individuals suffering from ABPA, an estimated 400,000 of these adults are also suffering from chronic pulmonary aspergillosis (CPA). In addition to CPA, there are various Aspergillosis diseases, which are caused by infection of fungi from the genus *Aspergillus.* These species do not typically cause infection in healthy individuals; however, these pathogens are incredibly dangerous for immunocompromised patients, which is generally the case for many fungal pathogens (Herbrecht et al. 2002). Moreover, many fungal infections are considered nosocomial diseases, meaning the pathogen originates in a healthcare setting, which heightens the severity of the mycoses threat for vulnerable patients (Schaberg et al. 1991). While many fungal infections are relatively minor and most immune systems are capable of preventing serious infection, fungal pathogens are still responsible for high rates of morbidity and mortality (Hope et al. 2013). A multitude of fungal opportunistic pathogens can establish infection when the body is vulnerable. When HIV/AIDS first emerged, 70% of the first four hundred recorded deaths were due to *Pneumocystis* pneumonia (PCP) (Armstrong-James et al. 2014). As of 2014, there are about 950,000 cases of

cryptococcal meningitis (CM) annually, thus accounting for the majority of the global burden of HIV-related fungal infections (Armstrong-James et al. 2014).

Recently, the threat of fungal infections in the United States has amplified due to the report of thirteen cases of *Candida auris* since May 2013 in four states: New York, Illinois, Maryland and New Jersey (CDC 2016). As of April 2017, the number of *C. auris* cases has escalated in New Jersey and New York from 1 to 15 and 3 to 44, respectively (CDC 2017). *C. auris* is a multi-drug resistant yeast that can cause life threatening fungal infections. Any infection caused by a yeast from the *Candida* genus is known as candidiasis; systemic infections are referred to as candidemia. *C. auris* is considered to have a stronger resistance than other pathogens from this species and it is much more difficult to identify. As a result, patients who acquire candidiasis, by *C. auris,* are likely to receive the wrong treatment due to misidentification. Moreover, *C. auris* spreads rapidly through clinical settings and persists on surfaces, once again heightening the threat to immunocompromised patients in healthcare facilities. Currently, an estimated 46,000 cases of candidemia occur each year in the US, resulting in about 220 deaths per year (CDC 2017). Patients who acquire candidemia during their hospital visit will spend another 3-13 days hospitalized, resulting in additional health care costs between $6,000-$29,000 (CDC 2016). Furthermore, once acquired during a hospital stay, an estimated 30% of patients will die due to this infection. As a result, the Center for Disease Control and Prevention (CDC 2016) has issued clinical alerts to health care facilities across the US, in order to help prevent a large-scale outbreak. The emergence of *C. auris* in the US is indicative of the increasing global threat of mycoses.

Current Anti-Fungal Drugs

The antifungal drug industry lacks agents that cause minimal side effects, due to the innate similarities between all eukaryotic organisms. As eukaryotes: mammals, plants, and fungi, all share similar molecular mechanisms, and as a consequence, most agents that are toxic to fungi are also toxic to the host. As a result, the availability of antifungals is underdeveloped in comparison to antibacterial drugs. Prokaryotic cells, most notably pathogenic bacteria, exhibit a greater amount of potential drug targets due to the large dissimilarity between prokaryotes and eukaryotes. Therefore, the unique structural and metabolic aspects of a bacterium can be targeted, leading to a lesser threat to the host's molecular machinery and biochemical processes. Just as antibacterial agents target unique pathogenic mechanisms, the pharmacological industry is in need of a target that is only present in fungal pathogens and absent from the infected host, despite their eukaryotic similarities.

The antifungal drug pipeline began in 1950 with the discovery of nystatin by Hazen and Brown. Nystatin effectively treated topical mycoses, in particular moniliasis, a yeast infection of the skin or mucous membrane (Hazen and Brown 1950). Due to the threat of systemic mycoses, researchers began to test for further applications of nystatin. Unfortunately, this drug could not be absorbed by the intestinal tract and intravenous administration of drug suspension caused dangerous side effects (Dutcher 1968). Thus, researchers aimed to find a drug with greater bioavailability to treat systemic mycoses. This pharmacological quest led to the discovery of amphotericin B by Squibb Laboratories (Dutcher 1968). The antifungal activity of amphotericin B was discovered while screening streptomycete cultures, specifically one that originated from soil found in the Orinoco Basin, Venezuela. More specifically, amphotericin B originates from *Streptomyces nodosus,* a filamentous bacterium, and targets the fungal cell membrane

component ergosterol (Purkait et al. 2011). Upon purification of the streptomycete culture, the antifungal substance was a combination of both amphotericin A and B. Bioassays and ultraviolet absorption spectrum indicated that amphotericin B was more potent than A and had a much greater inhibition activity than nystatin (Dutcher 1968).

Since its discovery, amphotericin B has been regarded as the gold standard of antifungals; however, the toxic side effects of the drug limit its use to only life-threatening infections (Mora-Duarte et al. 2002). Amphotericin B targets ergosterol, which is a main component of the fungal cell membrane; therefore, the drug binds tightly to this sterol and creates a pore in the membrane (Baginski et al. 2002). The pores caused by amphotericin B result in leakage of cellular components and eventual cell death. Unfortunately, ergosterol is the evolutionary precursor to cholesterol; thus, due to structural similarities, amphotericin B also binds cholesterol in cells of the human host. The affinity of amphotericin B for ergosterol is considered to be slightly higher than that of cholesterol; however, cholesterol is still targeted and this interaction is responsible for the drug's severe side effects (Baginski et al. 2002). The most common side effect is nephrotoxicity, which is toxicity of the kidneys leading to impairments in renal function (Mora-Duarte et al. 2002). The nephrotoxicity of amphotericin B was illustrated in a study conducted with patients who presented with invasive candidiasis, a yeast infection on the skin or mucous membrane. Of the patients who were treated with amphotericin B, 24.8% experienced a nephrotoxic effect (Mora-Duarte et al. 2002). In addition to nephrotoxicity, patients also experienced infusion-related effects such as chills, fever, tachycardia, tachypnea, hypertension, and nausea. The development of amphotericin B and the subsequent identification of its limitations spurred years of drug discovery research that continues into the present day.

The discovery of fluconazole, another widely used antifungal drug, was the result of a quest to find a drug that caused minimal toxicity and had a broader range of targets (Richardson et al. 1990). Since amphotericin B wreaked havoc on renal functions of infected patients, the industry was primed for the discovery of fluconazole. This drug is part of a class of azole molecules, which were previously known to exhibit antifungal properties (Richardson et al. 1990). Similar to amphotericin B, fluconazole targets ergosterol; however, the drug inhibits the synthesis of this molecule rather than the compound itself. Fluconazole was able to distribute throughout the tissues, was orally bioavailable, and had a lower risk for drug interactions and toxicity. As a result, fluconazole became one of the most widely used antifungals, specifically for AIDS patients suffering from oropharyngeal candidiasis (Richardson et al. 1990). However, this drug proved to be ineffective towards many opportunistic molds and some of the *Candida* species possessed a resistance. As the two of the first antifungal drugs, both amphotericin B and fluconazole illustrate the three challenges of the current antifungal armamentarium: adverse side effects/toxicity, multi-drug resistance, and limited spectrum agents.

In order to address the issue of limited spectrum drugs, a derivative of the triazole drug class was discovered: voriconazole (Herbrecht et al. 2002). This drug proved effective for immunocompromised patients suffering from invasive mold infections; thus, voriconazole was also administered as prophylaxis in hospital settings. In comparison to amphotericin B, voriconazole resulted in greater survival rates and fewer severe side effects when given to patients suffering from invasive Aspergillosis (Herbrecht et al. 2002). These diseases are particularly threatening to immunocompromised patients, specifically transplant recipients (Herbrecht et al. 2002). In a study published in the New England Journal of Medicine, a randomized clinical study was conducted with 277 (144 voriconazole, 133 amphotericin B)

patients with probable or definite Aspergillosis. Twelve weeks after treatment, 52.8% of the voriconazole group exhibited a successful response rate, in comparison to 31.6% of the amphotericin B group (Herbrecht et al. 2002). Moreover, patients treated with voriconazole experienced side effects, such as, hallucinations, blurred vision, and photophobia, which were considered less severe than those experienced by amphotericin B patients. Overall, 13.4% of voriconazole patients experienced these visual disturbances, while 24.3% of amphotericin B patients experienced renal impairments (Herbrecht et al. 2002). Unfortunately, the history of voriconazole illustrates some of the major issues of broader-spectrum agents. Specifically, voriconazole had a higher risk for drug interactions and increased pharmacokinetic variability. In regard to pharmacokinetic variability, the most common alterations are seen in drug absorption, distribution, metabolism, and excretion (Boucher et al. 2006). Therefore, it is difficult to administer the drug to maximum efficacy, without amplifying adverse effects. Overall, pharmacokinetic variability burdens a health care professional with having to closely monitor numerous physiological parameters in order to identify insidious changes in these factors.

One of the most promising discoveries in this industry during the 20th century was the development of echinocandin antifungal agents (Oakley et al. 1998). These drugs damage the fungal cell wall through noncompetitive inhibition of β-1,3-$_D$-glucan synthesis. Glucan is a major component of the fungal cell wall, yet not found in mammals. Therefore, the drug is effective with scant side effects; however, echinocandins lack activity against common opportunistic yeasts, most notably *Cryptococcus* species (Perlin 2007).

As illustrated throughout the antifungal drug pipeline, many of the currently used drugs cause sides effects that range from hallucinations to more severe effects, such as renal failure (Baginski et al. 2002). The flaws of these drugs exhibit the most challenging aspect of

pharmacological design: identifying fungal specific targets. Due to the innate similarities between all eukaryotes, the antifungal drug industry is in dire need of the identification of a fungal specific target, one of which is present in pathogens but absent from all potential hosts.

Agricultural Threat

Fungi also pose a significant threat to agriculture due to the danger of plant disease. Since there is a greater presence of resource-rich farming and microbial adaptation to new ecosystems, the fungal threat to agriculture is continually increasing (Fisher et al. 2013). The mounting adaptability of fungi is the result of trade and transportation, which results in the movement of these fungal species. Moreover, these pathogens are referred to as emerging infectious diseases (EIDs) due to their increasing incidence, virulence, and a broadened geographic or host range (Fisher et al. 2013). Fungal and other fungal-like pathogens threaten food security, most commonly: rice, wheat, maize, potatoes, and soybean (Fisher et al. 2013). Each crop is susceptible to the following pathogens, respectively, *Magnaporthe oryzae, Puccinia graminis, Ustilago maydis, Phytophthora infestans* and *Phakospora pachyrizi.* Although unlikely, if these pathogens simultaneously broke out in an epidemic, only 39% of the population would be left with adequate food supply (Fisher et al. 2013).

Phytophthora infestans

Potato crop is specifically threatened by *P. infestans,* a destructive oomycete, that causes the infection referred to as late potato blight. Oomycetes are eukaryotic microorganisms that are distinct from the fungi lineage. Some of the most destructive lower eukaryotic organisms originate from this oomycota class. Interestingly, *"Phytophthora"* means 'plant destroyer' (Birch and Whisson 2001). *P. infestans* was notoriously responsible for the mid-nineteenth century Irish potato famine (Haas et al. 2009). This began in the 1840s and caused major destruction to

Europe, the British Isles, and Ireland. Most of the damage was incurred in Ireland, where it was estimated that more than a quarter of this country's population diminished due to emigration and deaths caused by starvation (Birch and Whisson 2001). This Irish Potato Famine of Ireland has since been regarded as one of the most devastating crop diseases in history. In addition to potato crop, *P. infestans* also infects tomato and eggplant crops. *P. infestans* targets the foliage of these crops first, resulting in a yellow color that then turns black. Eventually the skin develops brown or purple spots that moisten, which is an ode to oomycetes' alternate names of "water mold" or "wet rot" (Birch and Whisson 2001). From the inception of infection, the crops quickly decay and spread the infection to neighboring crops, leading to larger agricultural destruction.

Today, potato is considered to be the fourth largest food crop worldwide (Haas et al. 2009), and thus losses incurred due to late potato blight are about $6.7 billion (Haverkort et al. 2008). This economic loss is also attributed to funding used for current fungicides, which are not effective enough to ensure food security. Due to its rapid adaptability, *P. infestans* has proven to be one of the most difficult crop pathogens to control (Haas et al. 2009). In comparison to other *Phytophthora* genomes, *P. infestans* possesses more extensive families of disease effector proteins, thus allowing for a greater damage to host health. Furthermore, the genes coding for these effector proteins are fast-evolving, thus most likely responsible for the pathogen's ability to adapt to its host. This rapid adaptability also allows *P. infestans* to develop resistance to many of the protective mechanisms that have been utilized over the years.

Molecular Genetics: DNA to RNA to Protein

Central Dogma of Molecular Biology

The Central Dogma of Molecular Biology, as proposed by Francis Crick in 1958, is the basis for understanding the process of protein translation (Crick 1970). Crick's declaration of the

process in which proteins are synthesized has become foundational knowledge for all molecular biologists. His ability to unite the complexities of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) to the intricate production of proteins has proven its fundamental importance. Genetic information essential for functioning, growth, development, and reproduction is encoded in DNA. RNA polymerases then transcribe this information into messenger RNA (mRNA) which is subsequently translated by ribosomes into protein.

DNA stores genetic information through linear sequences of nucleotides (Dever et al. 2016). There are four common bases that constitute the nucleotides of DNA: adenine (A), guanine (G), cytosine (C), and thymine (T). Distinct sequences of nucleotides form units of heredity, referred to as genes. Genes determine the order of a polypeptide chain, and thus protein structure and function. Nitrogenous bases pair together through hydrogen bonds, resulting in the antiparallel connection of two DNA strands, which then twists into a double helical structure. The DNA is further organized by wrapping around histones to create a complex that is referred to as nucleosomes. The organization of the genetic material using histones is known as the chromatin structure. In order to assure proper distribution during replication, chromatin is further condensed into chromosomes, reflecting the structural changes needed to protect the essential genetic instructions encoded in the DNA.

The final product, either a protein or RNA, that arises from information encoded in DNA begins with a process known as transcription (Dever et al. 2016). This primary process is initiated by the binding of protein factors, which may be responding to signals from within the cell, from another cell, or from changes in the environment. These protein factors bind to a specific region of a gene, the promoter, which signals the transcription machinery. After being

recruited to the start site, RNA polymerase synthesizes an mRNA strand that is complementary to one strand of DNA.

Eukaryotic Protein Translation

The mRNA that results from the transcription process relocates to the cytoplasm to deliver the genetic information to the ribosome, which then decodes the mRNA during the process known as translation (Dever et al. 2016). In order to decode the mRNA, the ribosome reads the codons to recruit transfer RNA (tRNA) with the anticodon sequences. A codon is a unit of genetic code, specifically three nucleotides, that corresponds to a specific amino acid. Amino acids are bound to tRNA molecules that present anticodon sequences corresponding to those presented on mRNA. The complementarity between the codon and anticodon allows for recruitment of the amino acid specified by the mRNA and when accomplished accurately, translation fidelity is attained. As the ribosome decodes the mRNA, the corresponding anticodon is delivered by tRNA, and the cognate amino acid is subsequently added to the polypeptide chain.

Protein translation is an essential process required for every cell to maintain viability. Overall, the entire process occurs within three steps: initiation, elongation, and termination (Dever et al. 2016). The first step is activated by the binding of the methionyl (Met)-transfer RNA $(tRNA)_i^{Met}$ to the 40S ribosomal subunit, which is already bound to mRNA. The 60S ribosomal subunit is recruited to the 40S, resulting in an established functional ribosome. The initiation step is completed by a total of 11 proteins, referred to as translation initiation factors (Dever et al. 2016). These factors' functions begin at the establishment of the preinitiation complex (PIC) and conclude with arranging the 80S ribosome to begin the next step in the translation. Following initiation, the ribosome carries out the codon-dependent addition of

amino acids in the elongation step of protein translation (**Figure 1**). The process involves all three binding pockets of the ribosome, known as aminoacyl-tRNA (A), peptidyl-tRNA (P), and exit (E). As the process reaches the elongation step, two canonical proteins are recruited, known as eukaryotic Elongation Factors (Klinge et al. 2012). Specifically, eukaryotic Elongation Factor 1A (eEF1A) binds and recruits the aminoacyl-tRNA (aa-tRNA) to the A site of the ribosome (Dever et al. 2016). aa-tRNA, or the charged tRNA, is bound to the cognate amino acid, which matches the anticodon of the tRNA. Upon peptide bond formation, the tRNA in the A site is transferred to the P site, and becomes the peptidyl tRNA. eEF1A recruits the next charged tRNA to the A site. Eukaryotic Elongation Factor 2 (eEF2), the second highly conserved translation elongation protein, translocates the 60S subunit, with respect to the 40S subunit, along the mRNA. This results in the movement of the tRNAs from the P and A site, to the E and P site. The tRNA in the E site is ready to be ejected, in order to allow the elongation cycle to continue. During the process of protein synthesis, only two of the three sites of the ribosome can be occupied at once; thus, the uncharged tRNA, which has already given its amino acid to the polypeptide chain, must be removed before eEF1A can recruit the next charged tRNA to the ribosome.

In addition to the canonical factors, eukaryotic Elongation Factor 3 (eEF3) has been identified as a third essential protein of this process in fungi; yet, eEF3 is absent in higher eukaryotes (Skogerson and Wakatama 1976). Specifically, eEF3 is considered to be responsible for removing uncharged tRNA from the E site of the ribosome through actions dependent on eEF1A interactions (Anand et al. 2003). eEF3, eEF1A and eEF2 work collaboratively to move the tRNAs between the pockets of the ribosome in order for the elongation step to function as a cycle. Moreover, in eukaryotes, the translation elongation process is then efficiently carried out

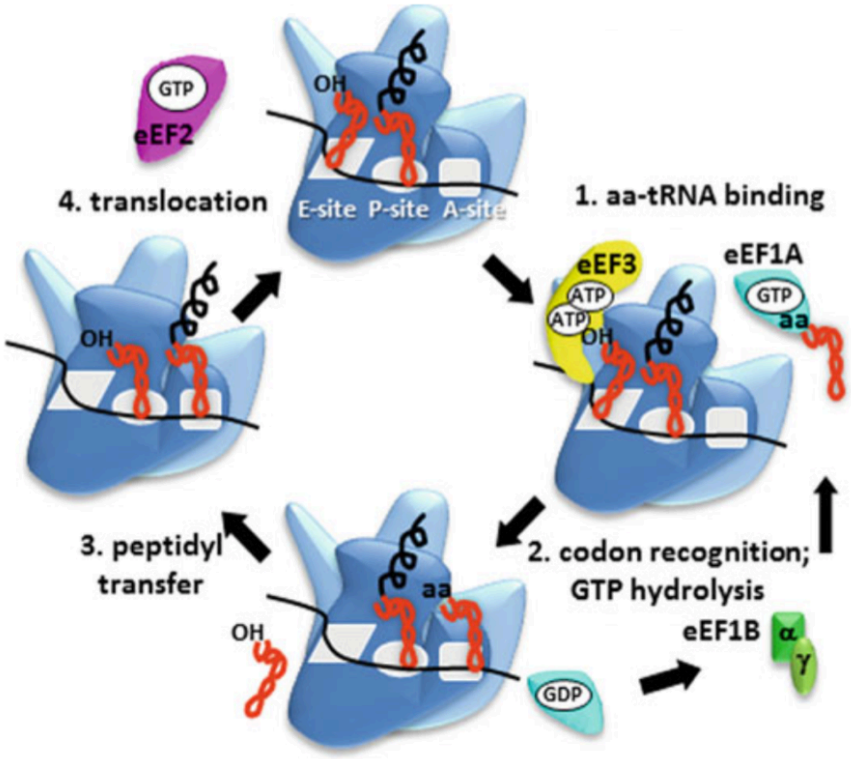


Figure 1 ***S. cerevisiae*** **(eukaryotic) Protein Translation: Elongation**. (Step 1) aa-tRNA is delivered to the A site of the ribosome by eEF1A. Simultaneously, eEF3 ejects deacylated tRNA from the E site. (Step 2) The GTPase activity of eEF1A is activated by the guanine nucleotide exchange factor (eEF1B) allowing for eEF1A release from the ribosome. (Step 3) The elongating polypeptide (shown as a black twisted line) is transferred to the P site upon peptide bond formation. (Step 4) eEF2 translocates the large ribosomal subunit, which results in the placement of the deacylated tRNA in the E site and the peptidyl tRNA in the P site. The cycle is then primed to begin again. Figure from Mateyak et al. 2016

through the use of polyribosomes, which allows for multiple ribosomes to translate the mRNA simultaneously, resulting in quicker protein production (Kopeina et al. 2008).

To conclude translation, the termination step is conducted, which is stimulated by the recognition of a stop codon in the A site (Dever et al. 2016). This recognition is accomplished by the effective interaction between eEF1A and eEF3. Specifically, eEF1A is responsible for the recognition of the stop codon and eEF3 facilitates the peptide release. Once the genetic information is translated from the mRNA into a polypeptide chain, it can then be properly folded into a protein.

Eukaryotic Elongation Factor 3 (eEF3)

While studying protein synthesis in *S. cerevisiae,* researchers Skogerson and Wakatama identified the third, previously unknown, elongation factor (1976). These researchers were conducting poly(U)-directed phenylalanine synthesis, which is a commonly used model system for studying protein synthesis. eEF1A and eEF2, already known elongation factors, were isolated from the system, along with the third factor. To demonstrate eEF3's essential role, eEF1A and eEF2 were used to catalyze protein synthesis in rat liver ribosomes (Skogerson and Engelhardt 1977). When rat liver ribosomes were replaced with yeast ribosomes, eEF3 was necessary to accomplish protein synthesis. This illustrates that the requirement of eEF3 is dependent upon the source of the ribosomes.

eEF3 has been identified as a protein that is recruited to the ribosomal translation complex during the elongation cycle (Anderson et al. 2006). eEF3's ability to bind to the ribosomal A site and to the aminoacyl-tRNA-eEF1A-GTP ternary complex is considered to be a required action during the translation cycle of fungi (Anderson et al. 2006). Research suggests that eEF3 is responsible for the ejection of the deacylated-tRNA from the ribosomal exit site

(Anand et al. 2003). This action is directly related to the function of eEF1A. eEF1A is responsible for delivering the cognate aminoacyl-tRNA to the ribosome, which is dependent upon E-site ejection (Figure 1). Considering only two of the three ribosomal binding pockets can be occupied at once, eEF3's role in removing the deacylated-tRNA is absolutely essential in order for translation elongation to continue its cycle to completion.

A significant protein domain of eEF3 is the ATPase Binding Cassettes (ABC). The ability of eEF3 to eject the deacylated-tRNA from the E site of the ribosome is suggested to be greatly dependent upon ABC hydrolytic activity (Anand et al. 2003). This is evidenced by the introduction of a mutation between the ATPase Binding Cassettes (ABC), the F650S mutation. This mutation replaced the amino acid phenylalanine to serine at the 650 location, which is where the two ABC motifs are connected. As a result of the F650S mutation in the *YEF3* gene (which encodes *S. cerevisiae* eEF3), eEF3 was incapable of hydrolyzing ATP. This was due to a change in the alignment of the ABC motifs, which further resulted in a global reduction in protein translation (Anand et al. 2003). Furthermore, this mutation resulted in decreased interactions between eEF3 and eEF1A. Thus, conservation of sequences between the ABCs is crucial to the overall function of the eEF3 protein and its interaction with other elongation factors.

Through understanding the structure of eEF3, a vulnerability has been identified. With the prospect of developing an antifungal agent to target eEF3, it is important to note that disrupting the connection between the ABCs would most likely cause a knockdown of this protein's overall function. However, due to the highly conserved nature of all ATPase domains, a greater understanding of eEF3's structure is necessary to design a highly specific drug that would target only the ABCs of this protein.

eEF3: A Potential Anti-fungal Target

eEF3, first discovered in *S. cerevisiae* (Skogerson and Wakatama 1976), was determined to be functionally conserved in *Candida albicans* (Colthurst et al. 1991), *Cryptococcus neoformans* (Blakely 2001) and *Schizosaccharomyces pombe* (Colmer 2013). These organisms all belong to the fungal kingdom; thus, eEF3 has been considered to be a fungal-specific protein, which is absent in mammals.

In recent years, the Dunaway laboratory has assessed the functional conservation of eEF3-like proteins from the fission yeast, *S. pombe* (Colmer 2013), and the green algae *Chlamydomonas reinhardtii* (Bourdot 2015). A former Dunaway laboratory member, Sarah Colmer, successfully cloned *S. pombe* eEF3 (SpEF3) into *S. cerevisiae* and exposed that strain to conditions that selected for the use of SpEF3 (Colmer 2013). Colmer conducted a plasmid shuffle scheme, as explicated in this study, and characterized cell viability in order to determine if ScEF3 could functionally complement for the loss of *S. cerevisiae*'s eEF3 (ScEF3). As the only source of eEF3 in this *S. cerevisiae* strain, SpEF3 was able to allow for cell viability, which indicates functional conservation of the eEF3 protein in *S. pombe.*

In order to assess further conservation of eEF3-like proteins outside the fungal kingdom, *C. reinhardtii* eEF3 was also studied in the Dunaway laboratory. As a single-celled green alga, *C. reinhardtii* is not part of the fungal kingdom. Kia Bourdot, a former Dunaway laboratory member, followed a similar experimental design; however, she found that *C. reinhardtii* (CrEF3) could not functionally complement for the loss of *S. cerevisiae* eEF3 (Bourdot 2015). While there are various reasons to explicate why CrEF3 could not complement for the loss of ScEF3, the result may be contributed to evidence that *C. reinhardtii* is lacking eEF1A. As previously noted, eEF1A is suggested to be essential to translation elongation in certain lower eukaryotic

organisms, specifically *S. cerevisiae,* as it is responsible for delivery of charged tRNA to the ribosome (Anand et al. 2003). In order for eEF1A to function properly, the deacylated tRNA must be removed by eEF3; thus, eEF3's proposed function is essential to the overall translation elongation process. Rather than the canonical eEF1A, *C. reinhardtii* utilizes a divergent factor, EF-like (EFL) (Keeling and Inagaki 2004). In regard to enzymatic activity, EFL and eEF1A are similar, as they are both members of the GTPase family; thus, EFL is capable of performing the translation-related functions of eEF1A. Moreover, these factors may be orthologues, which may have resulted from lateral gene transfer (Keeling and Inagaki 2004). Lateral gene transfer is a mechanism of evolution that refers to the exchange of genetic material, commonly between unicellular and/or multicellular organisms. By studying the evolutionary distribution of EFL, researchers found that its distribution among organisms appeared to be arbitrary (Keeling and Inagaki 2004); organisms possessing EFL are not necessarily closely related and many times absent from even the closest relatives. While the evolutionary distribution of EFL versus eEF1A remains unclear, the presence of one factor over the other contributes to understanding the evolutionary conservation of eEF3. The inability of CrEF3 to functionally complement, and the knowledge that this organism utilizes EFL instead of eEF1A contributed to the decision to study *P. infestans* eEF3, which expresses eEF1A.

In order for eEF3 to be confirmed as a putative antifungal treatment, a greater understanding of its conservation throughout lower eukaryotes is essential. Currently, the gaps in the antifungal treatments available are in part due to a lack of knowledge of possible evolutionary differences between pathogens (Blakely et al. 2001). Thus, it is crucial to identify how eEF3 is functionally conserved, in order to allow for the development of a broader application drug.

In order to further investigate the functional conservation of eEF3 outside the fungal kingdom, it was hypothesized that *P. infestans* eEF3 could complement for the loss of *S. cerevisiae's* endogenous eEF3 gene. This hypothesis was based on bioinformatics information that indicated the presence of an eEF3-like gene in *P. infestans*, the need for an agent to prevent late potato blight caused by *P. infestans*, and to challenge the four decade old notion that eEF3 is fungal specific. This was accomplished by cloning the *P. infestans* eEF3 gene and transforming the plasmid into *S. cerevisiae* to determine if it can provide the essential functions necessary for protein synthesis. By obtaining the answer to this question, we will be contributing to a larger quest of determining eEF3's functional conservation throughout the lower eukaryotic kingdom. The rapidly increasing threat of mycoses for immunocompromised individuals and the danger of plant pathogens to food supply further support the demand for this knowledge. The implication of this protein's necessity to lower eukaryotic cell viability will strengthen the foundation for a new drug target, which can have a broad application, with minimal collateral toxicity.

METHODS

Construction of *S. cerevisiae* Strains

Prior to my work in the Dunaway laboratory, *P. infestans* eEF3 (PiEF3) gene blocks were cloned into the *Escherichia coli* yeast expression vector pTKB328, which was previously generated by the Kinzy laboratory at Robert Wood Johnson Medical School (RWJMS). The pTKB328 vector, a centromeric (low copy) plasmid (Ortiz and Kinzy 2005), contains a leucine marker, polyhistidine-tag, and restriction sites for *Bam*HI, *Xho*I, and *Sac*I. The polyhistidine-tag, specifically 6XHis, is an amino acid motif of six histidines that are expressed on the N-terminus of proteins expressed on pTKB328. Therefore, cloning of PiEF3 in this plasmid allows for the

synthesis of 6XHis-tagged eEF3 expressed from the constitutive *TEF5* promoter (Ortiz and Kinzy 2005).

In order to assess the successful insertion of PiEF3, plasmid DNA was isolated using QIAGEN Qiaprep® Spin Miniprep Kit and digested with *Xho*I and *Sac*I endonucleases (New England BioLabs). The digested samples and a 1 kb marker (New England BioLabs #N3232) were run on a 1% agarose gel with 1X TAE buffer, and visualized using ethidium bromide. This diagnostic agarose gel was used to determine clones that contain PiEF3, which could be used for further experimentation.

In order to construct the SDY56, *S. cerevisiae* strain (**Table 1**), plasmids containing PiEF3 were transformed into *S. cerevisiae* strain TKY1617 (W3030 YEF3: His3 YEF3/PR5316), obtained from the Kinzy laboratory. Transformation was accomplished using the PEG-LiAc Yeast Transformation protocol (Gietz and Schiestl 2007). The *YEF3* gene, which encodes eEF3, had been previously deleted from the genome in the TKY1617 strain. Instead, *YEF3* is expressed on a plasmid containing a uracil marker, also known as the *URA3* gene.

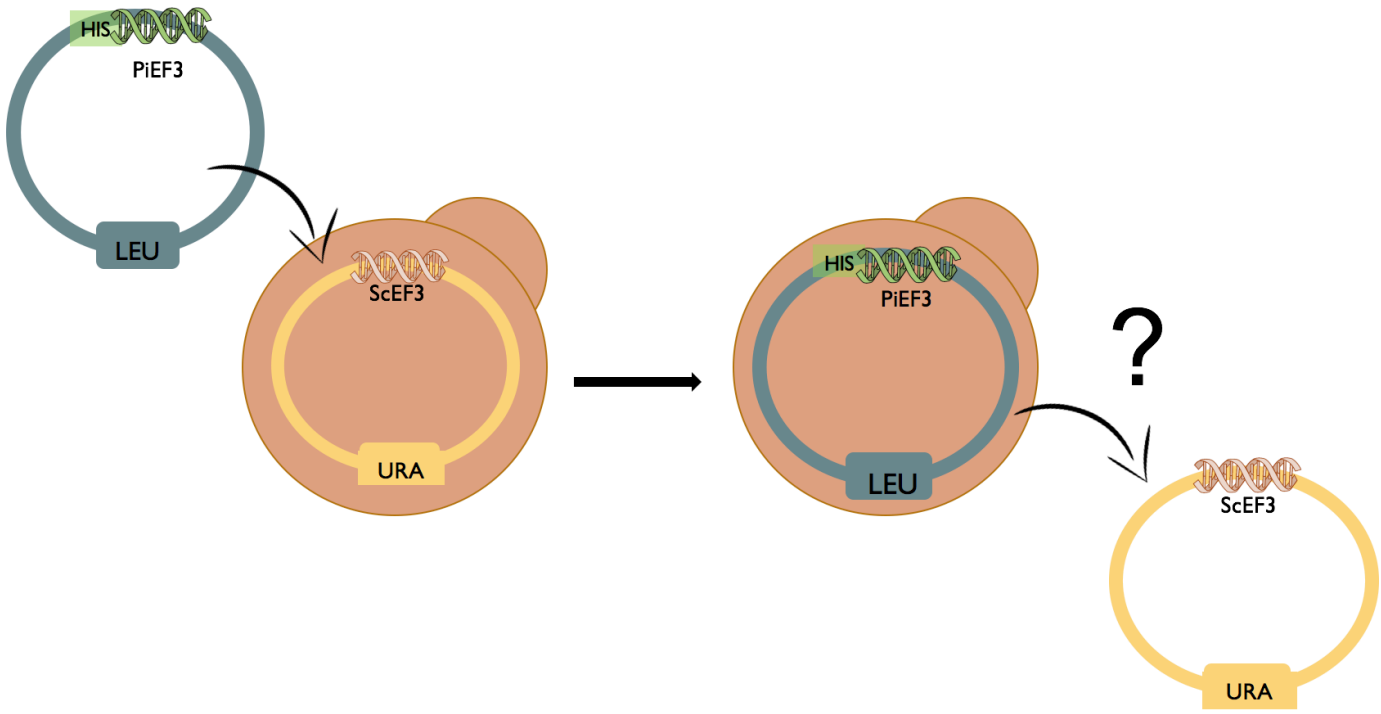
The Plasmid Shuffle

The plasmid shuffle is a genetic research tool that allows on to investigate whether orthologous genes can complement for one another (**Figure 2A)**. The PiEF3 containing plasmid has a gene that allows for the synthesis of the essential amino acid, leucine. The ScEF3 containing plasmid has a gene that allows for the synthesis of a different essential amino acid, uracil. As a result, when plated on selective media lacking one of these amino acids, the researcher can exploit the strains' abilities to synthesize these amino acids and select for one plasmid over the other. In this study, the PiEF3 (Leucine) containing plasmid was transformed

Table 1: *S. cerevisiae* strains details

Strain Number	Details	Genotype	Source
SDY56	*S. cerevisiae* expressing PiEF3	W3030 YEF3: His3 YEF3/PiEF3	Dunaway laboratory
SDY58	*S. cerevisiae* expressing ScEF3	W3030 YEF3: His3 YEF3/ScEF3	Dunaway laboratory
SDY59	*S. cerevisiae* expressing SpEF3	YF303 YEF3: His3 +YEF3+/SpEF3	Dunaway laboratory
TKY1617	*S. cerevisiae* expressing ScEF3 from *URA3* plasmid	W3030 YEF3: His3 YEF3/PR5316	Kinzy laboratory: RWJMS
TKY1717	N/A	W303	Kinzy laboratory: RWJMS

A



B

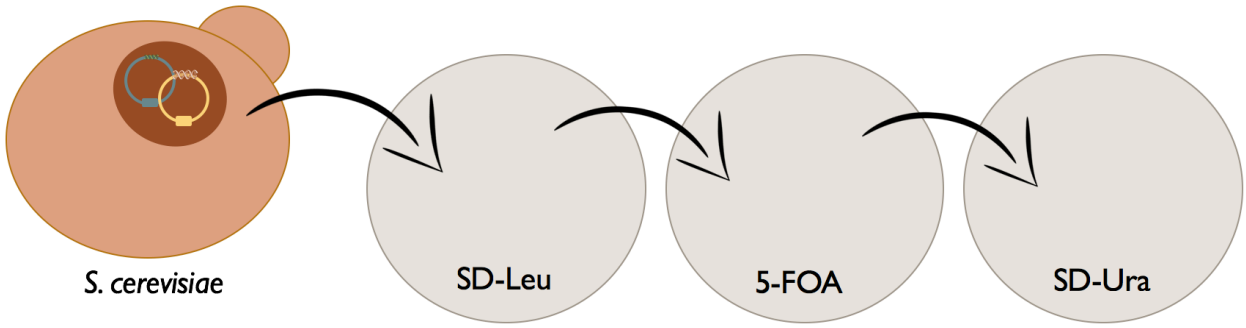


Figure 2 Plasmid Shuffle Scheme. (A) The TKB328 plasmid containing PiEF3 was transformed into *S. cerevisiae.* Our research question asks whether upon transformation, and due to selective plating, can *S. cerevisiae* use the PiEF3 plasmid and eject the plasmid containing its endogenous eEF3 (ScEF3)? **(B)** Selective plating was designed to force selection of PiEF3 plasmid and rejection of ScEF3, based on the nutritional markers present on these plasmids. SD-Leu media positively selects for the use of PiEF3 containing plasmid. 5-FOA negatively selects against cells using the ScEF3 plasmid. SD-Ura confirms loss of the ScEF3 plasmid.

into *S. cerevisiae* containing the ScEF3 (Uracil) containing plasmid. Following transformation, transformants were plated on selective media lacking the essential amino acid leucine (SD-Leu) (**Figure 2B)**. SD-Leu positively selects for cells utilizing the PiEF3 containing plasmid, because these cells must be synthesizing their own leucine. TKY1617, which expresses ScEF3 from the plasmid containing the uracil marker, was used as a negative control. The positive control strain, SDY59, was previously constructed by Sarah Colmer (Table 1). This *S. cerevisiae* strain expresses *Schizosaccharomyces pombe* eEF3 (SpEF3) from a plasmid containing the leucine marker. The SD-Leu surviving colonies were then plated on 5-Fluoroorotic Acid (5-FOA) media, which negatively selects against expression of the *URA3* gene and therefore expression of ScEF3, considering its presence on the same plasmid. The positive and negative controls for this step were the same as those used for SD-Leu plating. As the last step of the plasmid shuffle, 5-FOA surviving colonies were plated on selective media lacking the essential amino acid uracil; therefore, this step can confirm the loss of the ScEF3 (uracil) plasmid. *S. cerevisiae* expressing SpEF3 and TKY1617 were used as the negative and positive control, respectively.

For the succeeding experiments, the SDY58 strain was constructed to express endogenous *S. cerevisiae* eEF3 (ScEF3) on the same vector present in the other strains, rather than using a wildtype strain. This qualifies our results by eliminating variability, given that all eEF3 proteins studied are being expressed from the same promoter on a low copy plasmid which contains the 6XHis tag. The cloning of ScEF3 into pTKB328 was accomplished in the Kinzy laboratory; however, DNA purification and yeast transformation were completed in the Dunaway laboratory. Another *S. cerevisiae* strain of interest, TKY1717, was also obtained from the Kinzy laboratory. This strain lacks a 6XHis-tagged eEF3, thus it was utilized as a negative control in western blot analysis.

Expression levels of *P. infestans* eEF3 in *S. cerevisiae*

S. cerevisiae strains expressing ScEF3, SpEF3, or PiEF3 and the negative control strain TKY1717 were subjected to cell lysis via glass bead breakage by vortexing for 8 minutes at 4°C in a buffer containing 100 mM Tris (pH8), 20% glycerol, 1mM dithiothreitol [DTT], 1mM phenylmethylsulfonyl fluoride [PMSF], and a protease inhibitor tablet (Pierce, ThermoFisher Scientific). Centrifugation was used to pellet cellular debris, and the supernatant was retained. Protein concentration was determined using a Bradford Assay (Sigma Aldrich: Technical Bulletin). Bradford standards were prepared with bovine serum albumin (BSA) (Sigma Aldrich) concentrations ranging from 0-1.4 mg/mL; ultraviolet (UV) absorption was read at 595 nm using a BioMate 3 Spectrophotometer (ThermoFisher Scientific). Samples were prepared at a 1:10 dilution with Lysis buffer, then added to 1 mL of Bradford Reagent (Sigma Aldrich) to obtain net absorbance. Data were analyzed using Microsoft® Excel to determine a best-fit linear regression of the standards absorbance readings, and protein concentration was calculated in mg/mL for each sample from the slope.

All samples were prepared using gel sample buffer (100mM Tris-HCl pH6.8, 4% sodium dodecyl sulfate [SDS], 0.2% bromophenol blue, 20% glycerol, 200mM DTT) to obtain a final concentration of 1 μg/μL. Samples were loaded in a 10% Tris HCl gel (Bio-Rad), and run at 120 V for ~60 min in 1X Tris-glycine tank buffer (0.025 M Tris, 0.19 M glycine, 1% SDS). Proteins were transferred to a nitrocellulose membrane in methanol transfer buffer (25mM Tris base, 190mM glycine, 20% methanol) at 300 mA for 60 minutes. The membrane was blocked for one hour with TBST (2M Tris-Cl pH 8, 5M NaCl, 0.05% tween) with 5% powdered milk. Primary 6XHis antibody (BD Pharmingen, catalog number: 552565) was applied at a 1:10,000 dilution for one hour. The nitrocellulose membrane was washed and rinsed 3 times using TBST with 5%

milk before adding the secondary antibody. Goat anti-rabbit IgG, the secondary antibody (Santa Cruz Biotechnology), was added at a 1:5,000 dilution for one hour at room temperature. The nitrocellulose membrane was washed with TBST three times and developed using SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific). The western blot was auto-exposed on a Detection and Imaging Device (GE Healthcare Life Sciences).

Growth Analysis

S. cerevisiae strains expressing ScEF3, SpEF3, and PiEF3 PiEF3 were grown in yeast extract dextrose peptone dextrose (YEPD) liquid media at 30°C to an optical density (OD_{600}) of 2.0 on a BioMate 3 Spectrophotometer (ThermoFisher Scientific). Each sample was serially diluted ten-fold, then plated on YEPD using a Multi-Blot replicator (Machine shop: Rutgers University) and incubated at 30°C for 48 hours.

Drug Sensitivity Assay

To gain insight into growth defects, the sensitivity of the strains to known translation inhibiting drugs was analyzed. Liquid cultures were grown in YEPD media to an OD_{600} of 0.3. Cultures were plated on YEPD plates containing three 5-mm diameter filter discs. Each filter disc received 2 μL of one of the following antibiotics: cycloheximide (1mM), hygromycin (25mM), or paromomycin (800 mg/mL) (Sigma Aldrich). These concentrations were chosen based on a similar drug sensitivity assay completed by Anand and colleagues (2003). After 48 hours of incubation at 30˚C, the diameter of each zone of inhibition was measured in millimeters, by averaging the measurements of two diameter lengths, each in an opposite direction. Three trials of this assay were conducted with different starting samples in order to report an average inhibition zone for each strain in regard to all three drugs.

RESULTS

Successful Cloning of *P. infestans* eEF3 into *S. cerevisiae*

In order to confirm that *P. infestans* (PiEF3) was successfully inserted into the TKB328 plasmid, a double digest with *Bam*HI and *Xho*I was performed. If both insertion and digestion were successful, we expected to see a band at ~7 kilobase (kb) for the digested TKB328 plasmid, and a band released at ~3 kb for the PiEF3 insert, which is ~3129 bp. pTKB328-PiEF3 clones A and B were analyzed in a diagnostic agarose gel. A 1 kb marker (New England BioLabs) was also loaded for measurement (**Figure 3**, lane 5). For the undigested sample of pTKB328-PiEF3, a band is visualized at ~10 kb as well as bands above the intense band, which are nicked and linear versions of the plasmid (Figure 3, lane 1 and 3). A band is visualized at ~7 kb (Figure 3, lanes 2 and 4), which may indicate the presence of the pTKB328 plasmid alone, and a band at ~3 kb (Figure 3, lanes 2 and 4), which suggests the presence of the PiEF3 insert.

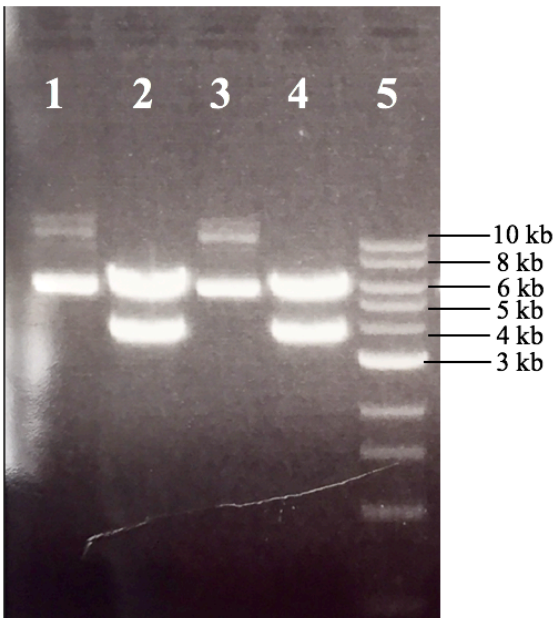


Figure 3 Diagnostic agarose gel suggests successful insertion of PiEF3 in pTKB328. Lane1, uncut pTKB328-PiEF3, clone A. Lane 2, double digested pTKB328-PiEF3, clone A. Lane 3, uncut pTKB328-PiEF3, clone B. Lane 4, double digested pTKB328-PiEF3, clone B. Lane 5, 1 kB ladder (New England BioLabs). All double digested samples were cut with *Bam*HI and *Xho*I.

Functional Complementation of PiEF3

In order to assess the ability of *P. infestans* eEF3 to complement for the loss of *S. cerevisiae* wildtype eEF3, PiEF3 was expressed in *S. cerevisiae* using the plasmid pTKB328. A plasmid shuffle scheme was employed in order to encourage the release of the plasmid containing the *S. cerevisiae*'s endogenous eEF3, and the subsequent use of PiEF3. Given its leucine nutritional marker, the PiEF3 plasmid can be selected for by plating on media lacking leucine (SD-Leu) following yeast transformation (**Figure 4**). Growth on SD-Leu suggests that the transformation of the plasmid containing the leucine marker was successful; therefore, the cells are capable of synthesizing this essential amino acid.

Cells from the yeast transformation were immediately plated on SD-Leu, and 72 hours later, two separate colonies (referred to as clone A and clone B) were restruck onto another SD-Leu plate. These strains are differentiated by Clone A and Clone B because they received two different PiEF3 containing plasmids, although both were confirmed for successful insertion through a diagnostic agarose gel and contain the same leucine marker (Figure 3). *S. cerevisiae* expressing SpEF3 was also plated on SD-Leu as a positive control, as it was previously shown to utilize the orthologous eEF3 and release the endogenous eEF3 plasmid (Colmer 2013); therefore, it must be capable of synthesizing its own leucine (Figure 4). The yeast strain TKY1617 was plated as a negative control; it harbors the uracil plasmid, and lacks a leucine nutritional marker needed to produce this amino acid when not provided in the media.

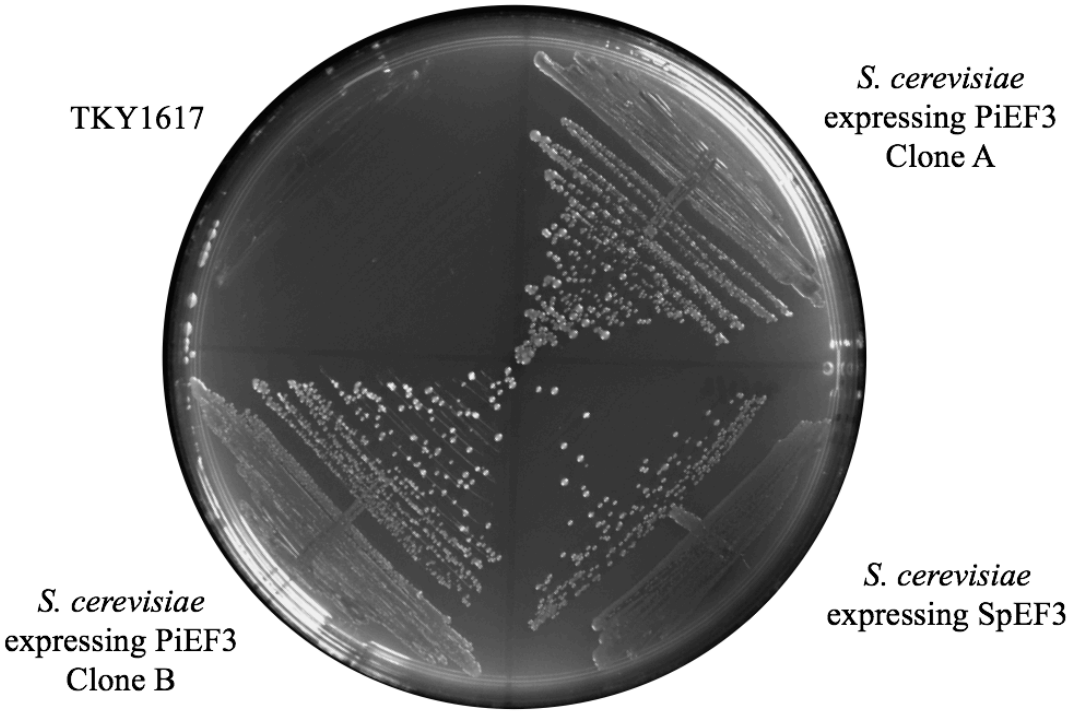


Figure 4 Growth on SD-Leu suggests use of the PiEF3 plasmid. *S. cerevisiae* strains expressing orthologous eEF3 (PiEF3 clone A, PiEF3 clone B, SpEF3) grow in the absence of leucine due to maintenance of the PiEF3 or SpEF3 plasmid, and thus the ability to produce their own leucine. TK1617 contains the *URA3* plasmid, and cannot produce its own leucine, which is necessary for growth on this plate.

In order to cause release of the plasmid containing the endogenous *S. cerevisiae* eEF3 (ScEF3), the cells were plated on 5-FOA media, as the next step of the plasmid shuffle. The ScEF3 plasmid contains a *URA3* gene; thus, plating on 5-FOA negatively selects for use of this plasmid. Specifically, in yeast strains expressing the *URA3* gene, 5-FOA is converted to toxic 5-flurouracil, resulting in cell death (Boeke et al. 1987). Growth on 5-FOA indicates that the cells have ejected the *URA3* containing plasmid; therefore, they are no longer expressing *S. cerevisiae* endogenous eEF3. Considering eEF3 is crucial to protein synthesis, and thus cell viability, it can be suggested that these 5-FOA resistant strains are expressing orthologous eEF3. The negative selection against the ScEF3 (uracil) plasmid have pressured the cells to release this plasmid and express orthologous eEF3 in order to survive.

Due to its previously determined loss of the *URA3* plasmid, *S. cerevisiae* expressing SpEF3 was used as a positive control and showed growth on 5-FOA media (**Figure 5**). As a negative control, TKY1617 did not grow on 5-FOA due to its use of the *URA3* plasmid and subsequent toxicity. The *S. cerevisiae* strains transformed with PiEF3 also successfully grew on the 5-FOA plate. This 5-FOA resistance indicates that the *URA3* plasmid was ejected and PiEF3 is functioning as the sole source of eEF3 to carry out protein synthesis and maintain cell viability. If PiEF3 was not able to perform the essential function of homologous eEF3 in this *S. cerevisiae* transformant, then these cells would harbor the *URA3* plasmid to use ScEF3 and not confer 5-FOA resistance; thus, growth on 5-FOA would not be observed.

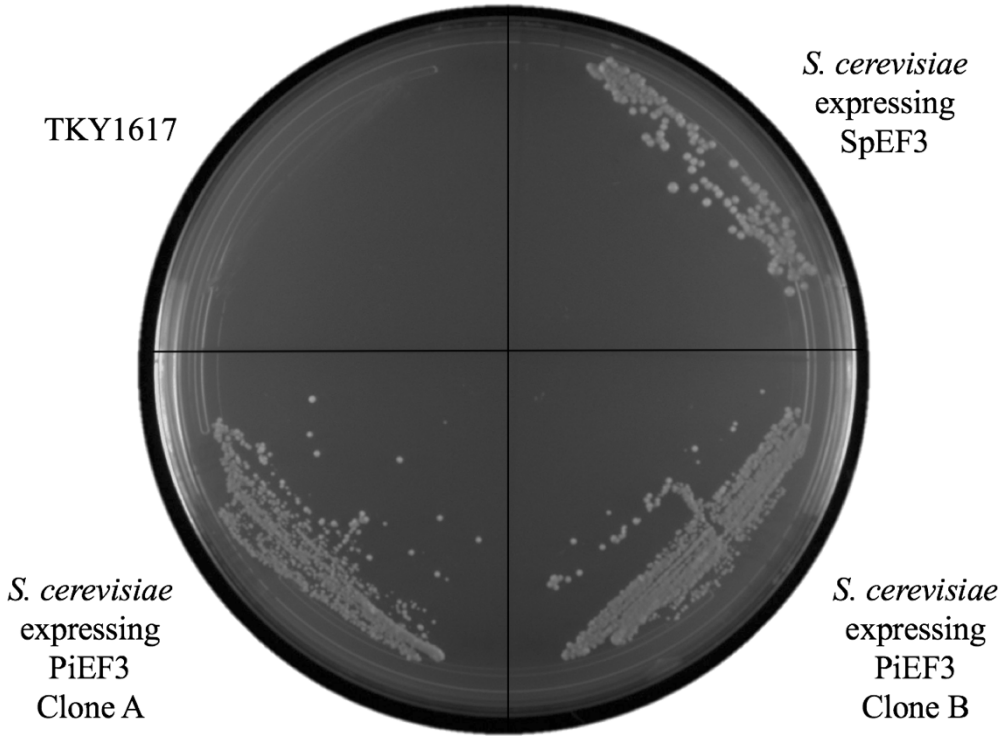


Figure 5 PiEF3 complements for the loss of *S. cerevisiae* endogenous eEF3. *S. cerevisiae* strains expressing orthologous eEF3 (PiEF3 clone A, PiEF3 clone B, SpEF3) grow in the presence of 5-FOA, whereas the wildtype strain does not. Phenotypic growth on 5-FOA monitors for the loss of the *S. cerevisiae* endogenous eEF3 plasmid, which contains the *URA3* gene responsible for cytotoxicity in the presence of 5-FOA. TKY1617 maintains the *URA3* plasmid.

As the last step of the plasmid shuffle, colonies from 5-FOA were restruck onto selective media lacking uracil (SD-Ura) to confirm loss of the *URA3* plasmid. TKY1617, a positive control, grows on SD-Ura because it maintained the *URA3* plasmid. *S. cerevisiae* expressing SpEF3 was used as a negative control since it was previously determined to utilize the SpEF3 plasmid and eject the *URA3* plasmid (Colmer 2013). The absence of growth for the *S. cerevisiae* strains expressing PiEF3 indicates that they have also released the *URA3* plasmid and are unable to produce their own uracil to maintain viability on media lacking this essential amino acid (**Figure 6**).

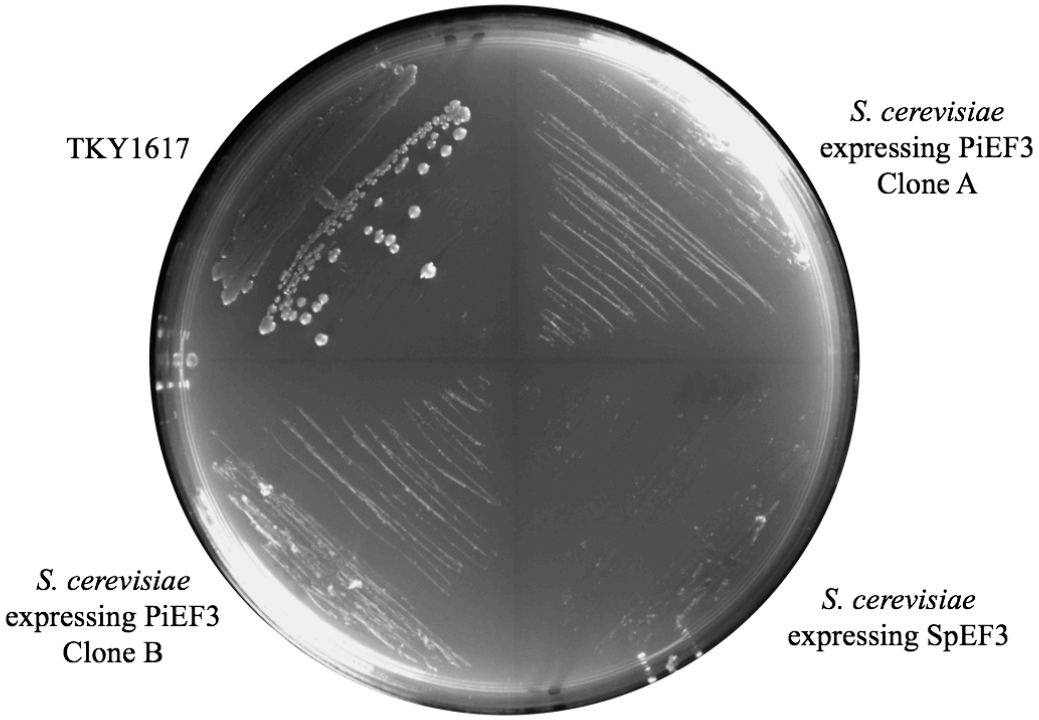


Figure 6 Absence of growth on SD-Ura indicates loss of ScEF3 plasmid. *S. cerevisiae* strains expressing orthologous eEF3 (PiEF3 clone A, PiEF3 clone B, SpEF3) do not grow in the absence of uracil due to ejection of ScEF3-carrying *URA3*- marked plasmid. TK1617 maintains the *URA3* plasmid and can produce its own uracil to needed for growth.

P. infestans 6XHis-tagged eEF3 is expressed at low levels in *S. cerevisiae*

Selection of the PiEF3 plasmid was further evaluated using western blot analysis, in order to determine the expression level of *P. infestans* eEF3 in *S. cerevisiae*. The eEF3 proteins studied in this experiment are 6XHis-tagged; therefore, eEF3 can be detected on a western blot using an anti-6XHis antibody. *S. cerevisiae* strains expressing ScEF3, SpEF3, or PiEF3, and the *S. cerevisiae* strain TKY1717 were analyzed (**Figure 7)**. An *E. coli* produced recombinant 6XHis positive control lysate was also loaded; according to BD Pharmingen, a band should be visualized ~30 kDa.

The 6XHis tagged-eEF3 proteins are expected to run at ~120 kDa. *S. cerevisiae* ScEF3 is expressing its endogenous eEF3; thus, its protein level was used for analysis as if this were the "wildtype" strain (Figure 7, lane 3). *S. cerevisiae* expressing SpEF3 was previously shown by Colmer to exhibit 6XHis-tagged eEF3, albeit at a lower level in comparison to when *S. cerevisiae* is expressing its endogenous protein (2013) (Figure 7, lane 4). The *S. cerevisiae* strain that was transformed with PiEF3 expresses this orthologous protein, although at a much lower level compared to *S. cerevisiae* expressing ScEF3 or SpEF3, indicated by decreased intensity of the band at approximately 120 kDa (Figure 7, Lane 5). Faint bands visualized above the protein of interest may be due to nonspecific binding. Based on these data, PiEF3 protein is present at a lower level, which may be due to instability of this protein considering it is not endogenous to the *S. cerevisiae* strain expressing it. Overall, PiEF3 is actively expressed by *S. cerevisiae* and complementing for the loss of the endogenous eEF3 (Figure 7 and Figure 5).

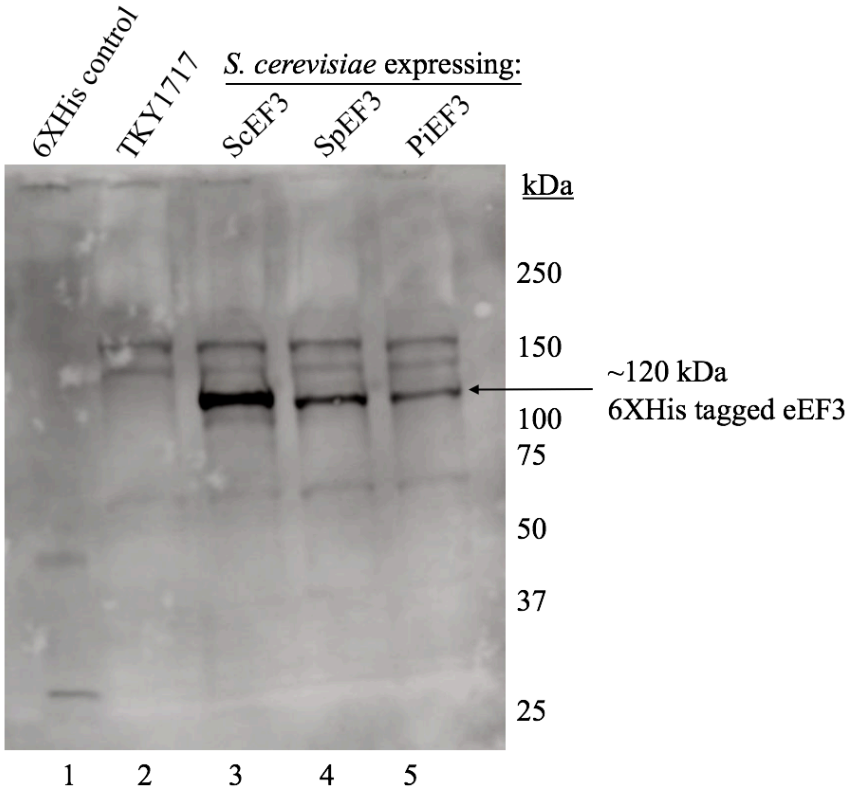


Figure 7 *P. infestans* 6XHis-tagged eEF3 is expressed at low levels in *S. cerevisiae*. Lane 1 contains an *E.coli* produced recombinant 6XHis control lysate; this is a positive control for the 6XHis antibody. Lane 2 contains the negative control *S. cerevisiae* strain (TKY1717) lacking a 6XHis tag, and lanes 3-5 contain *S. cerevisiae* expressing 6XHis tagged ScEF3, SpEF3, or PiEF3. 6XHis tagged eEF3 is expected at ~120 kDa.

Growth Analysis

In order to further analyze the cell viability of the *S. cerevisiae* strain using the orthologous PiEF3, a spotting assay was conducted. In this assay, each strain is plated at equally decreasing concentrations and the growth in each spot can be compared between strains to indicate possible growth retardation. Although our previous results indicate that the lowered levels of PiEF3 functionally complements for the loss of endogenous ScEF3, monitoring growth allows for a qualitative comparison of viability between the *S. cerevisiae* strains. We expected to see normal growth for *S. cerevisiae* expressing ScEF3, comparable to wildtype growth. Previously, *S. cerevisiae* expressing SpEF3 was shown to grow at a decreased rate as compared to the ScEF3 strain (Colmer 2013). Given the lower level of protein expression (Figure 7), it can be expected that *S. cerevisiae* expressing PiEF3 would illustrate the lowest rate of growth.

The spotting assay shows columns 1-5, which were plated by a 10-fold serial dilution; therefore, concentration is decreasing from left to right (**Figure 8**). Differences in growth between *S. cerevisiae* expressing ScEF3 versus PiEF3 can be seen as early as column 3; however, the difference in growth between the strains is most evident in columns 4 and 5 (Figure 8). Consistent with previous research, the *S. cerevisiae* expressing SpEF3 appears to grow at a slower rate than *S. cerevisiae* expressing ScEF3. *S. cerevisiae* expressing PiEF3 appears to grow even slower than the other *S. cerevisiae* expressing orthologous eEF3, which is indicated by smaller colonies, specifically in columns 4 and 5 (Figure 8). Based on these observations, *S. cerevisiae* expressing PiEF3 appears to exhibit a growth retardation, in comparison to the other strains; therefore, it was further hypothesized that this slow phenotypic growth may be due to low efficiency or defects in protein synthesis in the presence of orthologous eEF3.

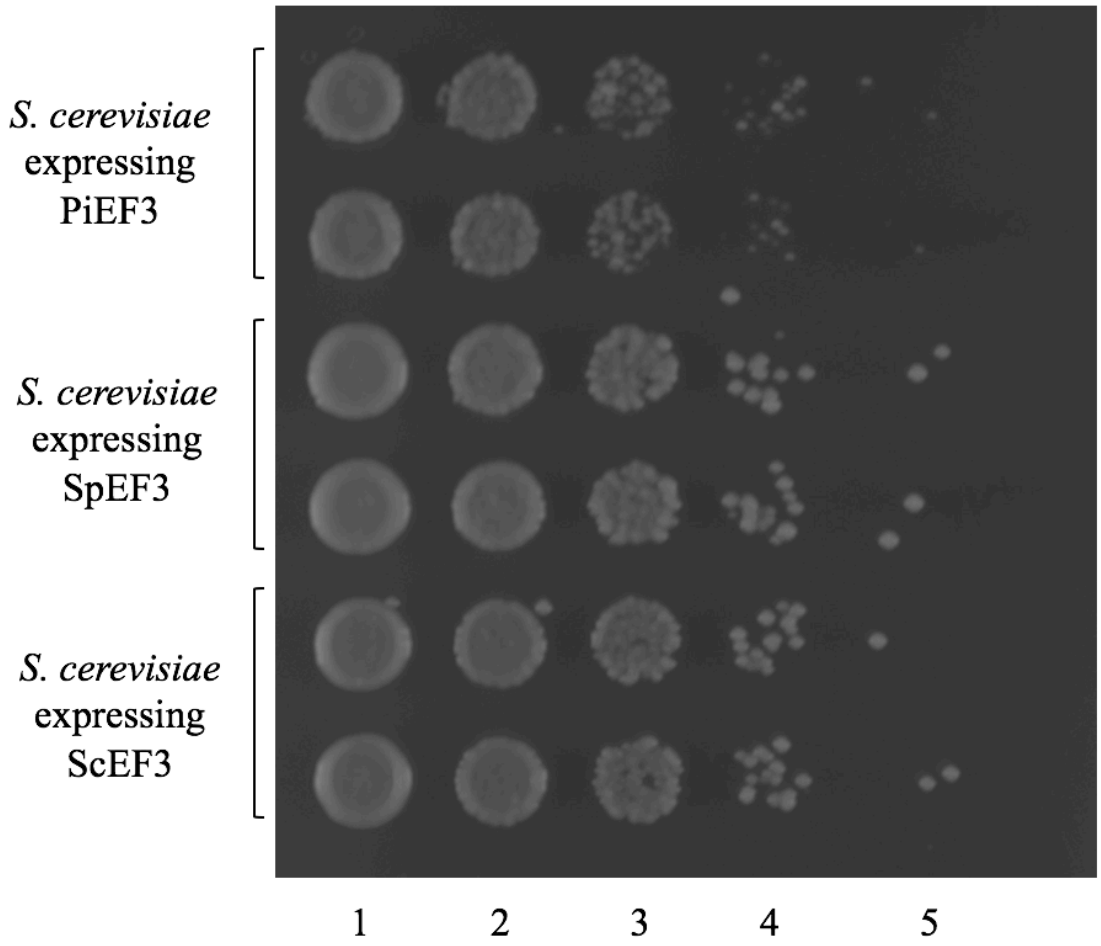


Figure 8 Growth curve analysis. *S. cerevisiae* strains expressing PiEF3, SpEF3, or ScEF3 were serially diluted by ten-fold, spotted on YEPD, and incubated at 30°C for 48 hours. Each strain was plated in duplicate from the same starting sample to allow for more than one observation.

Strain sensitivity to translation inhibitors

In order to analyze effects on protein synthesis in the *S. cerevisiae* strain expressing PiEF3, the strain's sensitivity to translation inhibiting drugs was tested. Due to the slower growth observed in the spotting assay (Figure 8) and the hypothesis that *S. cerevisiae* expressing PiEF3 exhibits defects in protein synthesis, translation inhibitors were expected to enhance these defects, indicated by increased drug sensitivity.

All three strains were exposed to cycloheximide, hygromycin, or paromomycin, which are known translation inhibitors (Blakely et al. 2001). Cycloheximide hinders translation elongation by binding to the large ribosomal subunit, specifically in the E site (Schneider-Poetsch et al. 2010). Hygromycin prevents translocation of the large ribosomal subunit during the elongation cycle (Blakely et al. 2001). Most notably, paromomycin binds to the 18S subunit of the ribosome to cause tRNA misreading and erroneous protein production (Lee et al. 2005). Comparing the strains' sensitivities to paromomycin was the most important aspect of this experiment. Based on previous research, paromomycin has the strongest correlation to defects in protein synthesis (Dinman and Kinzy 1997, Anand et al. 2003, and Fan-Minogue and Bedwell 2008) and there are various follow-up assays to characterize the sensitivity (Harger and Dinman 2003).

Each *S. cerevisiae* strain was plated on YEPD for a disc assay, in which each disc received one antibiotic. Average inhibition zones were determined by measuring two diameter lengths, one in each direction, as shown in **Figure 9**. The plate presented contains *S. cerevisiae* expressing PiEF3 along with inhibition zone halos and a representation of how each zone was measured (Figure 9).

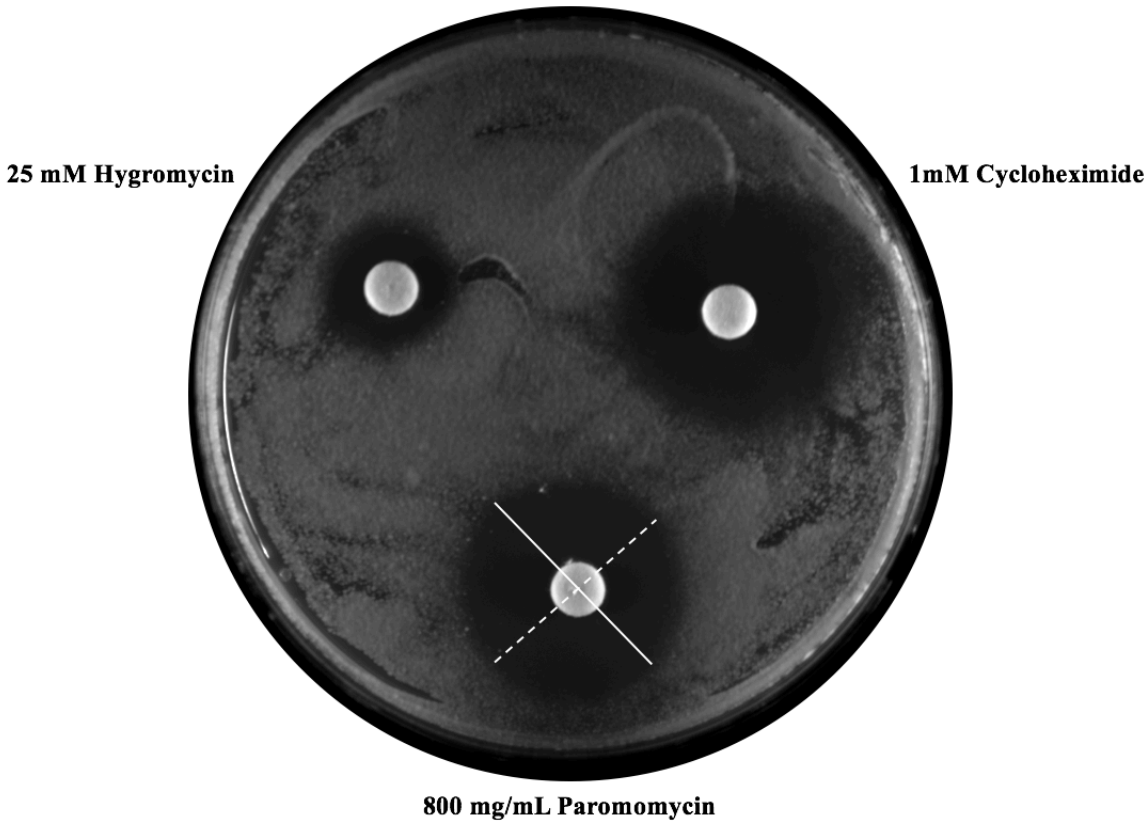


Figure 9 Representation of average inhibition zone measurement. *S. cerevisiae* expressing PiEF3 was plated on YEPD media with three discs, containing one of the known translation inhibiting drugs. Drug concentration was determined based on similar assay by Anand and colleagues (2003). The diameter of the halo was measured in one direction (solid line) and in the opposite direction (dotted line) and the values were averaged together.

The zones of inhibition, specific to strain and drug, are presented in **Figure 10**. When exposed to cycloheximide, *S. cerevisiae* expressing PiEF3 exhibited the largest inhibition zone, in comparison to smaller zones exhibited by SpEF3 and ScEF3; however, due to overlap in standard deviation a definitive conclusion cannot be made. In the presence of hygromycin, *S. cerevisiae* expressing ScEF3 or SpEF3 illustrated inhibition zones that were close in measure, while *S. cerevisiae* expressing PiEF3 has a zone that was almost 2 mm larger. The most evident variability was observed when the strains were exposed to paromomycin. *S. cerevisiae* expressing PiEF3 had an inhibition zone that was ~ 2-fold the diameter of *S. cerevisiae* expressing ScEF3. The inhibition zone of *S. cerevisiae* expressing SpEF3 measured between that of ScEF3 and PiEF3. This qualitative analysis suggests that *S. cerevisiae* expressing orthologous eEF3, specifically PiEF3, may experience defects in protein synthesis. Moreover, *S. cerevisiae* PiEF3 appeared to be most sensitive to paromomycin, in comparison to the two other strains. Paromomycin causes changes in the fidelity of the translation process, most likely causing frameshift mutations. Thus, it is hypothesized that PiEF3 functionally complements for ScEF3 to maintain viability, however, the strain may exhibit changes in translational fidelity due to defects in protein synthesis.

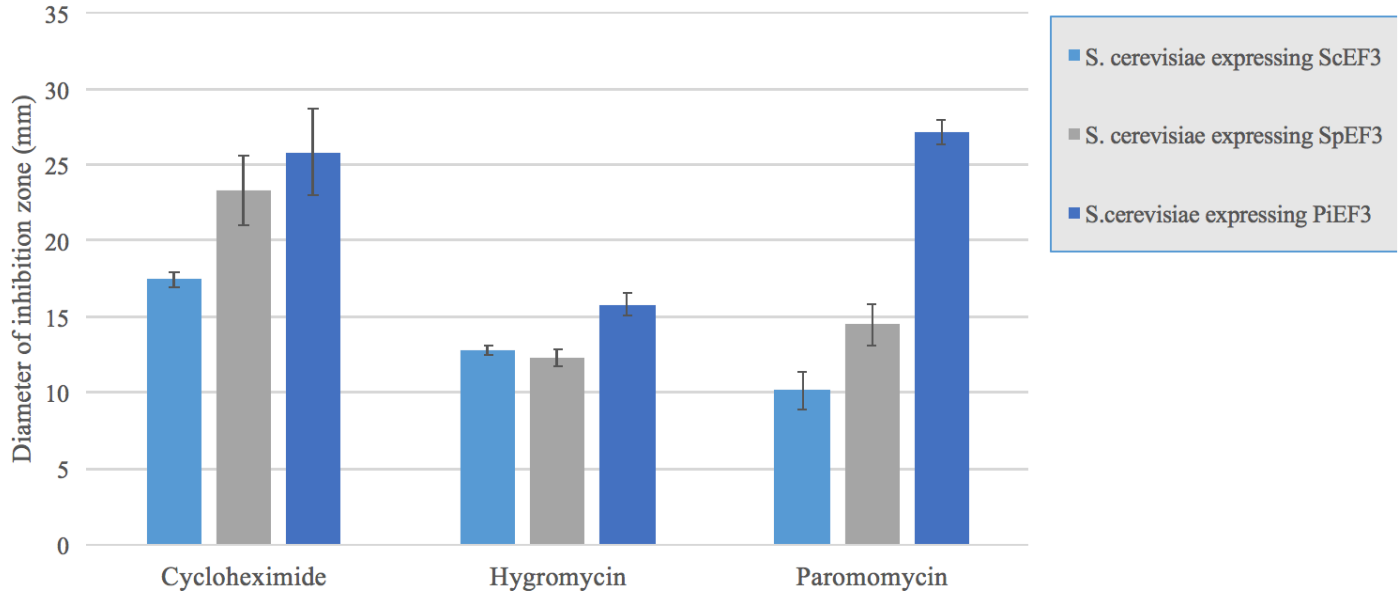


Figure 10 Sensitivity of *S. cerevisiae* strains to translation inhibitors. Cells were plated on YEPD and cycloheximide, hygromycin, or paromomycin were added to each filter disc. After 48 hours at 30ºC, the average diameter of the zones of inhibition were measured in mm. The recorded diameters are the average of two diameter measurements (two different directions) and the average of three experiments. Error bars represent the standard deviation of the measurements from all three trials.

DISCUSSION

As the danger of fungal infections continues to threaten health and agriculture, the need for identification of drug targets heightens. The discovery of eEF3 has offered a promising drug strategy, in which protein synthesis can be inhibited through targeting eEF3. With the implication that eEF3 is crucial to fungal viability, yet absent in higher eukaryotic organisms, targeting this protein should cause minimal collateral toxicity in mammalian hosts. Thus, understanding the conservation of this protein throughout the lower eukaryotic kingdom is of pharmacological importance. Previously, the conservation of eEF3 has been identified throughout the fungal kingdom, specifically in the Ascomycota and Basidiomycota divisions. The eEF3-like protein of *S. pombe*, an ascomycete yeast, was previously characterized by the Dunaway laboratory (Colmer 2013), and the eEF3 of *Cryptococcus neoformans*, a basidiomycete, was characterized by Blakely and colleagues (2001). The research presented in this study debunks the assertion that eEF3 is merely "fungal specific". As an oomycete, a lineage distinct from fungi, the conservation and characterization of *P. infestans* eEF3 suggests a wider conservation of this protein throughout the lower eukaryotic kingdom. Identifying a broader conservation of eEF3 allows for the effective design of pharmacological inhibitors that will be effective against a wide range of pathogens.

P. infestans and *S. cerevisiae* eEF3 proteins share ~62% similarity, and we hypothesized that this orthologous protein would complement for the loss of *S. cerevisiae* endogenous eEF3. Due to this shared similarity, it is highly likely that domains, such as the ATPase Binding Cassettes and motifs that allow for ribosome binding, are conserved in these eEF3 proteins. The orthologous eEF3 used in this study, PiEF3, was able to function as the sole source of eEF3 in *S. cerevisiae,* which may have been due to its conservation of these domains.

The results presented in this study indicate that PiEF3 complements for the loss; thus, suggesting eEF3 is functionally conserved outside the fungal kingdom. This conclusion challenges the 4-decade old notion that eEF3 is a fungal specific protein. The results of plasmid shuffle technique employed in this study were the preliminary indication that PiEF3 could support *S. cerevisiae* viability which successfully supports the initial hypothesis of this study. Specifically, this is implied due to growth on SD-Leu (Figure 4), growth on 5-FOA (Figure 5), and the absence of growth on SD-Ura (Figure 6). Upon further analysis, PiEF3 was in fact being expressed as shown in the western blot (Figure 7); thus, we could continue our studies to characterize PiEF3's complementation. *S. cerevisiae* expressing PiEF3 appeared to gorw sloers than *S. cerevisiae* expressing SpEF3, which was indicated in the spotting assay (Figure 8). Based on these results, we hypothesized that the observed growth defect was due to less efficient protein synthesis, since the strain was utilizing orthologous eEF3 rather than its homologous protein. In order to investigate this hypothesis, the sensitivity of the strains to translation inhibitors was assessed (Figure 9). Consistent with our hypothesis, the viability of *S. cerevisiae* expressing orthologous eEF3 was further compromised in the presence of translation inhibitors, specifically paromomycin (Figure 10), suggesting that these strains harbored defects in protein synthesis.

Based on the growth defects observed in our results, future research could focus on polyribosome analysis. Polysome profiling is an assay used to understand how well protein synthesis is being accomplished (Blakely et al. 2001) Through determining levels of the number of ribosomes translating a strand of mRNA, conclusions can be made regarding how well the ribosomes are functioning (Kopeina et al 2008). Due to the decreased level of PiEF3 expression and increased sensitivity to translation inhibitors, we would expect *S. cerevisiae* expressing

PiEF3 to exhibit defects in assembling multiple ribosomes on an mRNA strand. Furthermore, due to specific increased sensitivity to paromomycin, *S. cerevisiae* expressing PiEF3 is likely to exhibit negative changes in translational fidelity. Thus, a frameshift assay could be conducted in order to gain insight into potential translational infidelity (Harger and Dinman 2003). Frameshifting refers to changes in the open reading frame, resulting in altered translation of an mRNA sequence. As a translation inhibiting drug, paromomycin has specifically been shown to induce frameshifts (Lee et al. 2005). Due to the larger inhibition zones, *S. cerevisiae* expressing PiEF3 is more sensitive than *S. cerevisiae* expressing ScEF3 or SpEF3 to this drug, suggesting that this strain already may be experiencing translation infidelity and the presence of the drug is amplifying this defect.

In addition to gaining insight into protein translation defects, further research could be conducted to understand how the orthologous eEF3 is functioning with the *S. cerevisiae* ribosomes. This mechanistic data would investigate the hypothesis that PiEF3 would be stimulated less, in terms of ATPase activity, by the *S. cerevisiae* ribosome, in comparison to *S. cerevisiae* homologous eEF3. Since eEF3's function is dependent upon ATPase activity (Anand et al. 2003), measuring the activity in the presence of *S. cerevisiae* eEF3, versus a homologous ribosome, would be indicative of how well the orthologous eEF3 is able to function. When investigating the functional conservation of eEF3 in *C. neoformans,* Blakely and colleagues analyzed whether CnEF3 could be stimulated by *S. cerevisiae* ribosomes through measurement of ATPase activity (2001). Therefore, PiEF3 could be studied in a similar way in order to further characterize its complementation.

The observed slower growth (Figure 8) and increased sensitivity to translation inhibitors (Figure 10) may be due to the lower expression of PiEF3 (Figure 7). The Dunaway laboratory

will investigate this hypothesis further by cloning PiEF3 into a higher copy expression plasmid. If the observed defects, presented in this study, are simply due to the fact that orthologous eEF3 is being expressed at a lower level than homologous eEF3 is expressed, then we would expect these defects to be ameliorated when cells are expressing PiEF3 from the higher copy plasmid. This would imply that the eEF3 protein of *P. infestans* is more highly functionally conserved than our current results indicate; thus, bolstering the potential for an anti-eEF3 drug.

The implications of this study serve to shift the belief that eEF3 is a fungal-specific protein and provide further insight into the protein synthesis process of lower eukaryotic organisms. With eEF3 poised as a putative drug target, understanding the evolutionary distribution of this protein is essential to identifying how broad the potential drug's application will be. For the past 4 decades, eEF3 was considered to be a potential antifungal drug; however, these data imply that an anti-eEF3 drug will have wider application than what was originally believed. The potential presence of eEF3 outside the fungal kingdom, specifically in oomycetes, will allow for the development of pharmacological agents that would target pathogens that cause downy mildew, sudden oak death, stem and root rot, white rust, and many more threats to plants and food crops (Kamoun et al. 2015). Thus, studying the functional conservation of eEF3 will continue to identify pathogens that will be susceptible to an anti-eEF3 drug. Considering the intensifying threat of lower eukaryotic pathogens to both human and agricultural health, eEF3 will be a growing field of research with deeply rewarding implications.

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